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(54) **Repopulation of macrophages and granulocytes using transforming growth factor-beta.**

(57) Treatment with Transforming Growth Factor-β alone or in combination with a Colony Stimulating Factor therapeutically increases the number of granulocytes and monocyte/macrophages in mammals.

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Technical Field

This invention relates to the fields of chemotherapy and regulation of hematopoiesis. More specifically, this invention relates to methods for increasing macrophage and granulocyte populations in an individual, particularly after chemotherapy.

Background of the Invention

One of the primary distinguishing characteristics of cancer is the abnormally rapid division and proliferation of malignant cells. This is exploited in many current forms of chemotherapy, which have a greater effect on rapidly dividing cells than quiescent cells. Thus, normal, slowly dividing cells are less affected than malignant cells. Unfortunately, several cell types normally divide quickly, including the cells associated with hair follicles, intestinal lining, and hematopoiesis. The depression of hematopoiesis following chemotherapy is the most serious side effect, and in many cases leaves the patient susceptible to opportunistic infection due to the relative lack of lymphocytes, macrophages and granulocytes.

There are several known growth factors which are believed necessary for the induction and terminal differentiation of myeloid cells. Granulocyte-macrophage colony stimulating factor (GM-CSF) stimulates the formation of blast cells capable of differentiating into granulocytes (neutrophils and eosinophils) and macrophage/monocytes. Granulocyte colony stimulating factor (G-CSF) stimulates the production of granulocytes (predominantly neutrophils) from granulocyte/macrophage precursor cells. Macrophage colony stimulating factor (M-CSF) stimulates the production of monocyte/macrophages from granulocyte/macrophage precursor cells. Although it might seem a natural solution to administer GM-CSF, G-CSF, and/or M-CSF to patients undergoing chemotherapy, these cytokines may be effective only at toxic doses.

Seyedin, U.S. Pat. No. 4,774,322 filed 10 December 1987, described two bovine bone-derived cartilage inducing factors (CIFs), designated CIF-A and CIF-B. Both have molecular weights of approximately 26,000 daltons by SDS-PAGE and are dimers. They each exhibit *in vitro* chondrogenic activity by themselves, as measured by cartilage specific proteoglycan (PG) production in an agarose gel culture model using fetal rat mesenchymal cells. Neither, however, is chondrogenically active *in vivo* by itself. Amino acid sequencing of the CIF-A showed that it has a partial (30 amino acids) N-terminal sequence identical to that reported for a human placenta-derived polypeptide called beta-type transforming growth factor (TGF-β). The partial N-terminal sequence of CIF-B is different from that of TGF-β. Both CIFs exhibit activity in the TGF-β assay (ability to induce anchorage-independent growth of normal rat kidney cell colonies in soft agar). CIF-A/TGF-β is now known by the name TGF-β1, while CIF-B is now generally referred to as TGF-β2.

TGF-β, when combined with EGF or TGF-alpha, (1) promotes cell proliferation in the soft agar culture assay and (2) promotes cell proliferation and protein deposition in a rat soft tissue wound healing model. The applications characterize the TGF-βs as being dimers having a molecular weight of approximately 26,000 daltons (26 kDa) by SDS-PAGE. TGF-β exhibits a wide variety of activities, which appear to depend in large part to the cell type which is exposed. For example, TGF-β stimulates growth of normal fibroblasts in the presence of EGF or TGF-α, but inhibits the growth of some tumor cells: A.B. Roberts et al, Proc Nat Acad Sci USA (1985) 82:119-23. Bentz et al, U.S. Pat. No. 4,806,523, disclosed the use of TGF-β (also known as CIF-A and CIF-B) to inhibit inflammation, and demonstrated that TGF-β inhibited T-cell proliferation and antibody production. S. Tsunawaki et al, Nature (1988) 334:260-62 found that macrophages incubated with TGF-β1 or TGF-β2 were reversibly deactivated. H. Goey et al, J Immunol (1989) 143: 877-80 reported that administration of TGF-β1 directly to femoral bone marrow in mice *in vivo* reversibly suppressed the proliferation of pluripotential progenitor cells.

Disclosure of the Invention

We have now invented a method for treating mammals in need of increased numbers of granulocytes and macrophages, by administering TGF-β alone or in combination with a CSF. When TGF-β is administered preceding or concurrently with a CSF, the effective dose required of CSF is reduced to subtoxic levels.

Brief Description of the Drawings

Figure 1 depicts the increase in number of macrophages derived from bone marrow precursor cells of mice following *in vivo* administration of TGF-β1 followed by *ex vivo* administration of M-CSF, expressed as a percentage of control. The experiment is described in Example 1 below.

Figure 2 depicts the increase in number of macrophages and granulocytes derived from bone marrow pre-

cursor cells of mice following *in vivo* administration of TGF-β1 followed by *ex vivo* administration of GM-CSF, expressed as a percentage of control. The experiment is described in Example 1 below.

Figure 3 depicts the dose-dependent increase in number of macrophages and granulocytes derived from bone marrow precursor cells of mice following *in vivo* administration of TGF-β1 followed by *ex vivo* administration of GM-CSF, expressed as a percentage of control. The experiment is described in Example 2(B) below.

Modes of Carrying Out The Invention

A. Definitions

The term "TGF-β" or "transforming growth factor β" refers to the proteins TGF-β1, TGF-β2, and related proteins which are capable of binding to TGF-β receptors and effecting TGF-β type activity. In general, TGF-β activity may be assessed by the observation of anchorage independent growth in fibroblasts *in vitro*, and the promotion of protein and connective tissue formation in wound healing models *in vivo*. "TGF-β" includes all TGF-β-like proteins, whereas "TGF-β1" and TGF-β2" indicate only those specific proteins as described under the names CIF-A and CIF-B in U.S. Pat. No. 4,774,322, incorporated herein by reference.

The term "CSF" refers to a colony stimulating factor, and includes proteins which are capable of inducing progenitor cells to differentiate toward granulocyte and macrophage/monocyte cell types. The presently preferred CSFs are M-CSF, GM-CSF, and G-CSF.

"Macrophage colony stimulating factor" or M-CSF is a heavily glycosylated homodimer of about 45 kDa which stimulates the growth of macrophage/monocyte cells from the appropriate precursor cells. Purification of M-CSF (also known as CSF-1) from natural sources was described by E.R. Stanley, Meth Enzymol (1985) 116:564-87, while cloning was disclosed by E.S. Kawasaki et al, Science (1985) 230:291-96; and Kawasaki et al, PCT WO86/04607.

"Granulocyte-macrophage colony stimulating factor" or GM-CSF is a glycoprotein of about 23 kDa which stimulates the growth of cells capable of differentiation to granulocytes (including neutrophils and eosinophils) and macrophage/monocyte cells from the appropriate precursor cells.

"Granulocyte colony stimulating factor" or G-CSF is a glycoprotein having a molecular weight of about 18 kDa, which stimulates the proliferation of neutrophil colonies. The partial N-terminal sequence is Thr-Pro-Leu-Gly-Pro-Ala-Ser-Ser-Leu-Pro-Gln-Ser-Phe-Leu-Leu-Lys-Cys-Leu-Glu-Gln. Purification of G-CSF from conditioned media is described by Ono et al, U.S. Patent No. 4,833,127. Cloning and expression of G-CSF is described by Souza, U.S. Pat. No. 4,810,643.

B. General Method

TGF-β, GM-CSF, G-CSF, and M-CSF are all available from commercial sources or may be prepared from readily available materials using methods known to those skilled in the art, cited above.

TGF-β and the selected CSF(s) are used either *in vivo* or *ex vivo*. TGF-β is preferably administered *in vivo*. When administered *in vivo*, the proteins are generally introduced by parenteral routes, for example by intravenous injection, injection into the bone marrow, implanted device, intranasal aerosol, and the like. The presently preferred mode of *in vivo* administration is by intravenous injection.

The method of the invention is useful in any case wherein increased numbers of granulocytes and/or macrophages are desired, for example as the result of an immunosuppressive disorder, in treatment of infection or malignancy, or following treatment with agents having a myelosuppressive effect, whether intended or as a side effect. Suppression of granulopoiesis and myelopoiesis often occurs as a side effect in the treatment of malignancy with radiation or chemotherapeutic agents. This leaves the subject with an impaired ability to resist infection, often permitting opportunistic organisms to proliferate to a dangerous degree. In the practice of the invention, TGF-β is administered near or following the time of treatment or disorder: preferably TGF-β is administered between zero and five days following treatment. CSF is preferably administered only after chemotherapeutic or radiotherapeutic treatment, and is administered simultaneously with TGF-β or up to four days later. CSF administration preferably follows TGF-β administration by about 24-48 hours.

In the *ex vivo* methods of the invention, bone marrow (or other hematopoietic tissue) is removed from the subject (preferably) after administration of TGF-β, and the cells maintained in culture until treated with CSF.

Compositions of TGF-β, CSF, or combinations thereof for administration will generally include an effective amount of TGF-β and/or CSF in addition to a pharmaceutically acceptable excipient. Suitable excipients include most carriers approved for parenteral administration, including water, saline, Ringer's solution, Hank's solution, and solutions of glucose, lactose, dextrose, ethanol, glycerol, albumin, and the like. These compositions may optionally include stabilizers, antioxidants, antimicrobials, preservatives, buffering agents, surfactants, and

other accessory additives. A presently preferred vehicle comprises about 1 mg/mL serum albumin in phosphate-buffered saline (PBS). A thorough discussion of suitable vehicles for parenteral administration may be found in E.W. Martin, "Remington's Pharmaceutical Sciences" (Mack Pub. Co., current edition).

The precise dosage necessary will vary with the age, size, and condition of the subject, the nature and severity of the disorder to be treated, and the like: thus, a precise effective amount cannot be specified in advance. However, appropriate amounts may be determined by routine experimentation with animal models, as described below. In general terms, an effective dose of TGF-β will range from about 10 µg/Kg to about 1 mg/Kg. The presently preferred TGF-β is TGF-β1. An effective dose for M-CSF, GM-CSF, and/or G-CSF is about 10 µg/Kg to about 1 mg/Kg. Presently preferred CSFs are M-CSF and GM-CSF.

Suitable animal models include the mouse model illustrated in the Examples below. Briefly, TGF-β is administered parenterally, followed 1-9 days later with extraction of the bone marrow. The marrow is diluted to a single-cell suspension and treated with CSF. The treated cells are allowed to proliferate and differentiate into macrophages and/or granulocytes, and the degree of proliferation and differentiation assayed by standard techniques (e.g., peroxide generation, ^{3}H-thymidine incorporation, fluorescence-activated cell sorting (FACS), cell staining, and the like). The increased number (compared to controls) of granulocytes and/or macrophage/monocytes that results indicates the increased sensitivity to CSF by those precursor cells and may indicate increased number of precursor cells present due to TGF-β.

C. Examples

The examples presented below are provided as a further guide to the practitioner of ordinary skill in the art, and are not to be construed as limiting the invention in any way.

Example 1

(Demonstration of Activity)

(A) Natural bovine TGF-β1 was dissolved in 0.25 mL of 4 mM HCl, then 0.75 mL of mouse serum albumin (MSA, 1 mg/mL) in phosphate buffered saline (PBS) added to make a 500 µg/mL TGF-β1 stock solution. From this solution, three experimental solutions were prepared by diluting the stock to 25 µg/mL, 50 µg/mL, and 125 µg/mL. A control solution was prepared identically, but omitting the TGF-β1.

(B) Sixty female C57Bl mice were divided into 5 experimental groups (12 mice per group) and injected subcutaneously with TGF-β1 (0.2 mL) on Day 0 as follows:

A: 5 µg TGF-β1 per mouse;
B: 10 µg TGF-β1 per mouse;
C: 25 µg TGF-β1 per mouse;
D: untreated;
E: treated with diluent.

On day 1, 3 mice from each group were sacrificed, and bone marrow cells collected from the femur and tibia. This procedure is repeated on days 2, 4 and 9. Single cell suspensions were prepared in DMEM + 10% horse serum and adjusted to 10^5 cells/mL, and plated in 24-well plates at 1.0 mL suspension/well. To each well was then added M-CSF (0.1 mL of a 500 U/mL stock), GM-CSF (0.1 mL of a 500 U/mL stock), or 0.1 mL PBS. The plates were then incubated for 6 days in a CO_2 humidified incubator, then washed with PBS and the cells fixed with formaldehyde. The fixed plates were washed with 0.1 M borate buffer (pH 8.5), and stained with 1% methylene blue for 10 min. The stained plates were again washed with borate buffer, and allowed to air-dry. The stain was then eluted from the plates using 0.1 N HCl for 20 min at 42°C, and the absorption of the eluent read at 630 nm.

The results are shown in Figures 1 and 2. Figure 1 shows the increase in macrophage population resulting from the application of M-CSF to TGF-β1-induced marrow cells. Administration of 25 µg/mouse TGF-β1 increased macrophage number to about 240% of control on Day 1. This indicates that the number of macrophage precursor cells in the bone marrow was increased by the *in vivo* administration of TGF-β1. Figure 2 illustrates the increased number of macrophages and granulocytes obtained by administration of GM-CSF, indicating the increased sensitivity GM precursor cells in bone marrow to CSF due to the *in vivo* administration of TGF-β1. The control groups were not affected.

Example 2

(A) Fifteen male $CB6F_1$ mice were divided into 5 experimental groups (3 mice per group) and injected subcutaneously with TGF-β1 (0.2 mL) on Day 0 as follows:

A: Untreated;

B: diluent control;
C: 25 μg TGF-β1 per mouse;
D: 10 μg TGF-β1 per mouse;
E: 5 μg TGF-β1 per mouse.

On day 2, the mice from each group were sacrificed, and bone marrow cells collected from the femur and tibia. Single cell suspensions were prepared in DMEM + 10% horse serum and adjusted to 10^5 cells/mL, and plated in 24-well plates at 1.0 mL suspension/well. Then, M-CSF (0.1 mL of a 500 U/mL stock), GM-CSF (0.1 mL of a 500 U/mL stock), or 0.1 mL PBS was added to each well. The plates were then incubated for 6 days in a CO_2 humidified incubator, then washed with PBS and the cells fixed with formaldehyde. The fixed plates were washed with 0.1 M borate buffer (pH 8.5), and stained with 1% methylene blue for 10 min. The stained plates were again washed with borate buffer, and allowed to air-dry. The stain was then eluted from the plates using 0.1 N HCl for 20 min at 42°C, and the absorption of the eluent read at 630 nm.

The results are shown in Table 1 below. The results demonstrate that administration of TGF-β1 increased the sensitivity of macrophage and granulocyte precursor cells in bone marrow to CSF.

TABLE 1:

Group	% Untreated Control	% Diluent Control
A: Untreated	100.0	-
B: Diluent	100.0	-
+ M-CSF	88.7	-
+ GM-CSF	114.6	-
C: 25 μg TGF-β1		
+ M-CSF	161.3	181.9
+ GM-CSF	201.6	175.8
D: 10 μg TGF-β1		
+ M-CSF	166.4	187.6
+ GM-CSF	193	168.3
E: 5 μg TGF-β1		
+ M-CSF	177.5	200.2
+ GM-CSF	218.8	190.8

(B) The experiment of part (A) above was repeated, substituting doses of 1.0, 2.5, and 5.0 μg/mouse TGF-β. The results are shown in Figure 3, expressed as percent of control. A dose-dependent response was observed.

Example 3

(Mode of Administration)

This experiment was performed to determine if the mode of administration affected the biological response.

Twelve male $CB6F_1$ mice were divided into 5 experimental groups (3 mice per group) and injected subcutaneously with TGF-β1 (0.2 mL) on Day 0 as follows:

A: Untreated;
B: 25 μg TGF-β1 per mouse, subcutaneous;
C: 25 μg TGF-β1 per mouse, intravenous;
D: 25 μg TGF-β1 per mouse, intraperitoneal.

On day 2, the mice from each group were sacrificed, and bone marrow cells collected from the femur and tibia. Single cell suspensions were prepared in DMEM + 10% horse serum and adjusted to 10^5 cells/mL, and plated in 24-well plates at 1.0 mL suspension/well. M-CSF (0.1 mL of a 500 U/mL stock), GM-CSF (0.1 mL of a 500 U/mL stock), or 0.1 mL PBS was added to each well. The plates were then incubated for 6 days in a CO_2 humidified incubator, then washed with PBS and the cells fixed with formaldehyde. The fixed plates were washed with 0.1 M borate buffer (pH 8.5), and stained with 1% methylene blue for 10 min. The stained plates

were again washed with borate buffer, and allowed to air-dry. The stain was then eluted from the plates using 0.1 N HCl for 20 min at 42°C, and the absorption of the eluent read at 630 nm.

The results are shown in Table 2 below. The results demonstrate that the biological effect is independent of route of administration.

TABLE 2:

Group	% Untreated Control
A: Untreated	100.0
B: subcutaneous (thigh)	
+ M-CSF	194.7
+ GM-CSF	195.2
C: intravenous	
+ M-CSF	217
+ GM-CSF	213.5
D: intraperitoneal	
+ M-CSF	218.1
+ GM-CSF	214.4

Claims

1. A composition for inducing proliferation and differentiation of monocyte/macrophages or granulocytes, which composition comprises: TGF-β and CSF in admixture with one another or packaged separately from one another.

2. A composition according to claim 1, wherein said CSF is selected from M-CSF, G-CSF, and GM-CSF.

3. A composition according to claim 1 or 2 wherein said TGF-β is TGF-β1.

4. A composition according to any one of the preceding claims which composition comprises:
   a first container having an effective amount of TGF-β; and
   a second container having an effective amount of CSF.

5. A composition as defined in any one of the preceding claims for use in a method for inducing the proliferation and differentiation of mammalian granulocytes or monolyte/macrophages in a human or animal host by:
   contacting a hematopoietic stem cell with an effective amount of TGF-β; and
   contacting said cell with an effective amount of CSF within 0-5 days from said TGF-β contact.

6. A composition according to claim 5 wherein in the method, said cell is contacted with CSF abut 1-2 days after TGF-β contact.

7. A composition according to claim wherein, in the method, said cell is contacted with TGF-β in vivo by administering an effective amount of TGF-β to a host having a hematopoietic stem cell.

8. A composition according to claim 7 wherein the method further comprises:
   removing said hematopoietic cell from said mammal after contacting said cell with TGF-β, prior to contacting said cell with CSF, wherein said cell is contacted with CSF ex vivo.

9. A composition according to claim 8 wherein the method further comprises introducing said cell into a mammal in need of additional monocyte/macrophages or granulocytes, following contacting said cell with CSF.

10. A composition according to claim 7 wherein in the method said cell is contacted with CSF by administering an effective amount of CSF to said mammal in vivo.

11. A method for inducing the proliferation and differentiation of mammalian granulocytes or monocyte/macrophages which includes the step of ex vivo treatment with CSF of hematopoietic stem cells that have been treated with TGF-β.

12. A method according to claim 11 wherein the CSF treatment stem cells are returned to a host different from the host from which they were originally removed.

Figure 1

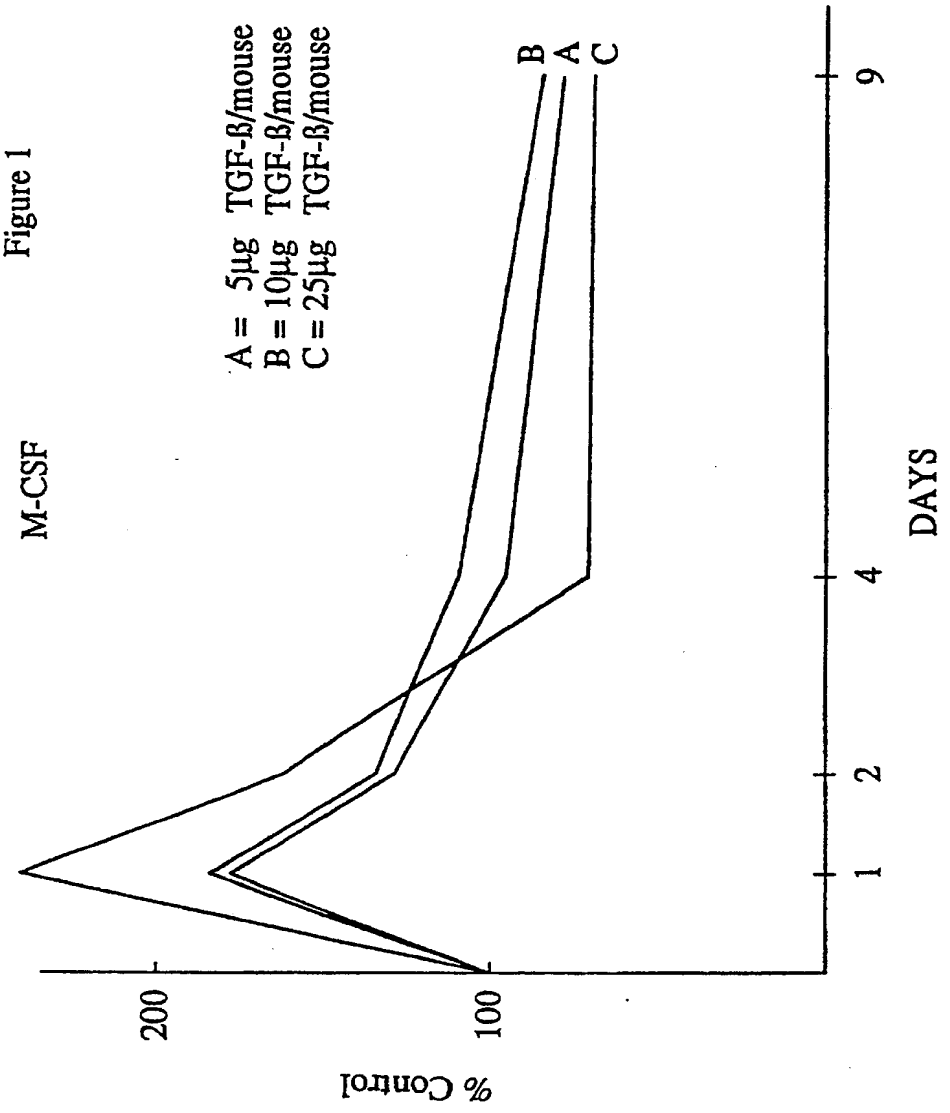

M-CSF
A = 5µg TGF-ß/mouse
B = 10µg TGF-ß/mouse
C = 25µg TGF-ß/mouse
% Control
200
100
1
2
4
9
DAYS
B
A
C

Figure 2

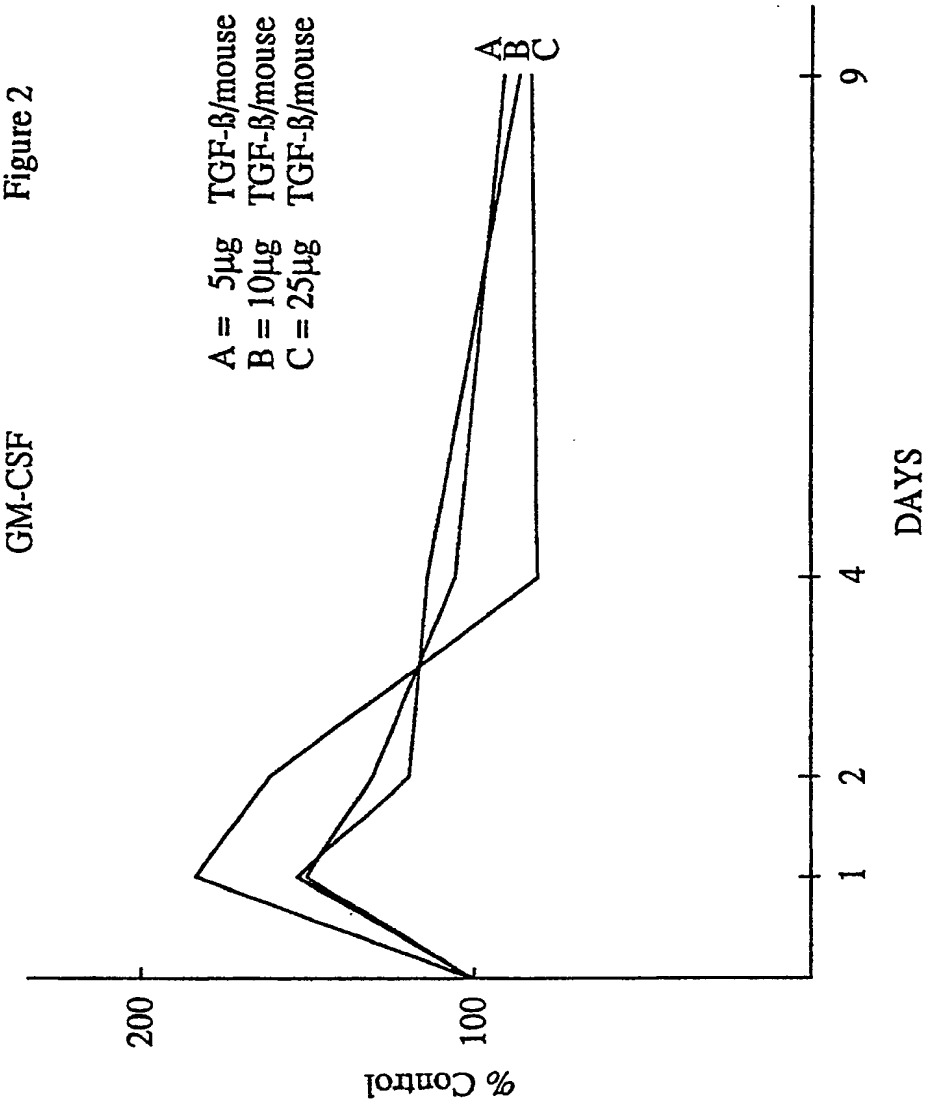
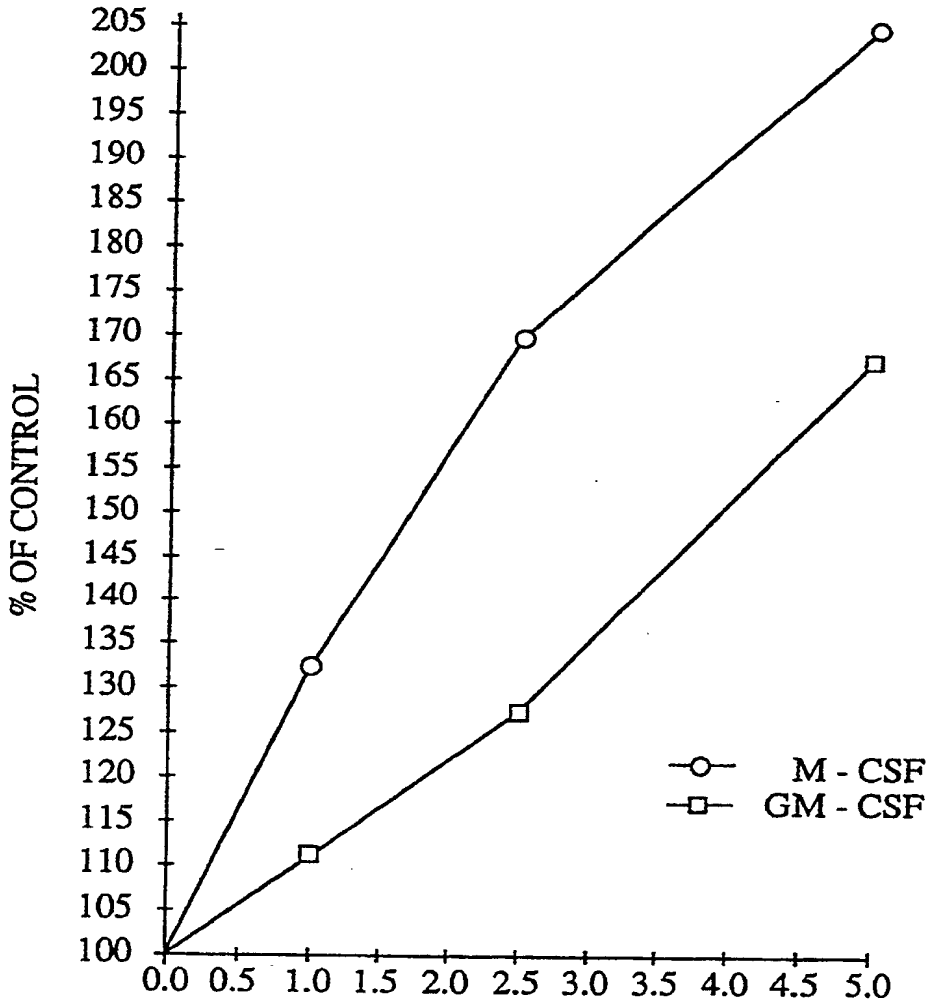

GM-CSF
A = 5µg TGF-ß/mouse
B = 10µg TGF-ß/mouse
C = 25µg TGF-ß/mouse
A
B
C
200
100
% Control
1
2
4
9
DAYS

Figure 3


205
200
195
190
185
180
175
170
165
160
155
150
145
140
135
130
125
120
115
110
105
100
% OF CONTROL
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0
TGF-BETA 1 (µg/mouse)
M - CSF
GM - CSF

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PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 91 30 3603

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	EXPERIMENTAL HEMATOLOGY, vol. 17, no. 3, March 1989, pages 296-299, New York, US; M. AGLIETTA et al.: "Interaction of transforming growth factor-beta 1 with hemopoietic growth factors in the regulation of human normal and leukemic myelopoiesis" * Abstract * -----	1-12	A 61 K 37/02 A 61 K 35/14

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

A 61 K

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

Remark: Although claim 12 is directed to a method of treatment of the animal/human body, the search has been carried out and based on the alleged effects of the compound/ composition.

Place of search	Date of completion of the search	Examiner
THE HAGUE	28-07-1992	NOOIJ F.J.M.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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